

Restoration of myocardial β -adrenergic receptor signaling after left ventricular assist device support

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Objective: Left ventricular assist device support for patients with chronic heart failure can significantly improve β -adrenergic receptor signaling, which is likely critical to myocardial recovery. The mechanism underlying the restoration of β -adrenergic receptor signaling is unclear. This study investigates our hypothesis that restoration of cardiac β -adrenergic receptor signaling by left ventricular assist devices results from inhibition of the G protein–coupled receptor kinase-2, a G protein–coupled receptor kinase that specifically phosphorylates and desensitizes agonist-occupied β -adrenergic receptors.

Methods: Left ventricular β -adrenergic receptor signaling was assessed in biopsy specimens taken from patients with chronic heart failure ($n = 12$) at the time of left ventricular assist device implantation (heart failure group) and again at the time of heart transplantation (left ventricular assist device group). Signaling was also studied in left ventricular biopsy specimens from nonfailing control ($n = 8$) hearts (nonfailing control group). Signaling was assessed by measuring sarcolemmal membrane β -adrenergic receptor density, adenylyl cyclase activity, G protein expression, and G protein–coupled receptor kinase-2 expression and activity.

Results: Left ventricular β -adrenergic receptor signaling was severely decreased in the heart failure group versus that seen in the nonfailing control group, as demonstrated by adenylyl cyclase activity. G protein–coupled receptor kinase-2 expression and activity was increased 3-fold in the heart failure group versus that seen in the nonfailing control group. After left ventricular assist device support, β -adrenergic receptor signaling was restored to levels similar to those seen in the nonfailing control group. G protein–coupled receptor kinase-2 expression and activity were markedly diminished after left ventricular assist device support compared with that seen in the heart failure group and were not different from that seen in the nonfailing control group.

Conclusion: In chronic heart failure left ventricular assist device support leads to restoration of cardiac β -adrenergic receptor signaling. The primary mechanism appears to be diminished myocardial G protein–coupled receptor kinase-2 activity. This demonstrates the potentially beneficial effects of G protein–coupled receptor kinase-2 inhibition on β -adrenergic receptor signaling in heart failure and might represent a novel therapeutic strategy for this disease process.

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Left ventricular assist devices (LVADs) have become increasingly important as a therapeutic option for patients with end-stage heart failure (HF). In addition to the significant clinical benefits provided by LVADs, many basic scientific observations have been made regarding several important myocardial signaling pathways in HF. LVADs can reverse many of the molecular, cellular, and neurohormonal abnormalities characteristic of advanced HF.¹⁻⁶ When the heart fails, a constellation of biochemical defects has been noted that includes significant alterations in the β -adrenergic receptor (β AR) signaling system. Dysfunctional

Abbreviations and Acronyms

AC	= adenylyl cyclase
ANOVA	= analysis of variance
ATP	= adenosine triphosphate
β AR	= β -adrenergic receptor
β ARK1	= β -adrenergic receptor kinase-1
B_{\max}	= total β AR density
cAMP	= cyclic adenosine monophosphate
DU	= densitometry units
GRK	= G protein-coupled receptor kinase
HF	= heart failure
LVAD	= left ventricular assist device
NF group	= nonfailing group

β AR signaling in HF includes receptor downregulation and impaired signaling through remaining receptors, which is known as desensitization.⁷⁻⁹ HF is associated with an increased level of circulating catecholamines and an approximately 3-fold increase in activity of the G protein-coupled receptor kinase-2 (GRK2).^{10,11} GRK2, also known as β AR kinase-1 (β ARK1), is a member of the GRK family of serine-threonine kinases and can phosphorylate and desensitize agonist-occupied β ARs.¹² It has recently been shown that LVAD support restores β AR responsiveness and reverses receptor downregulation in failing human hearts.¹³ However, the mechanism underlying this restoration of signaling is unclear. In addition to the hemodynamic effects of mechanical unloading, it appears that some aspects of reverse remodeling might also depend on normalization of the neurohormonal milieu.^{1,14}

We hypothesized that the primary mechanism of restoration of left ventricular β AR signaling after LVAD support is a decrease in myocardial GRK2 activity leading to up-regulation and improved signaling through β ARs. GRK2 has been shown to be a critical modulator of cardiac function in vivo,^{15,16} and inhibition of GRK2 activity as a result of LVAD support might play an important role in the potential recovery of ventricular function in end-stage HF.

Materials and Method**Myocardial Tissue Collection**

The left ventricular apical core excised during implantation of the HeartMate XVE LVAS (Thoratec) for each patient was snap-frozen in liquid nitrogen and stored at -80°C . A section of the anterior wall of the left ventricle was then excised and stored in identical fashion after LVAD explantation and cardiectomy at the time of heart transplantation. All samples were paired from LVAD implantation to transplantation ($n = 12$). Nonfailing control left ventricular apical tissue was obtained from organ donors whose hearts were unsuitable for transplantation but who had normal ventricular function and no structural heart disease ($n = 8$).

Protein Immunoblotting

Tissue was homogenized in ice-cold lysis buffer (25 mmol/L Tris-HCl [pH 7.5], 5 mmol/L ethylenediamine tetraacetic acid, 5 mmol/L ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid, 10 $\mu\text{g}/\text{mL}$ leupeptin, 20 $\mu\text{g}/\text{mL}$ aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride). Nuclei and tissue were separated by means of centrifugation at 800g for 20 minutes. The crude supernatant was then centrifuged at 20,000g for 20 minutes. Protein concentrations were determined on the supernatant (cytosolic fraction). Sedimented proteins (membrane fraction) were resuspended in 50 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (pH 7.3) and 5 mmol/L MgCl_2 . The immunodetection of myocardial levels of β ARK1 (GRK2, polyclonal rabbit IgG; Santa Cruz Biotechnology, Inc) was performed on an equal amount of protein from cytosolic and membrane extracts (80 μg) electrophoresed through 12% Tris-glycine gels and transferred to nitrocellulose. Membranes were blocked in 5% nonfat dried milk in 0.1% Tween 20 in phosphate-buffered saline for 1 hour at room temperature. The protein was visualized by using a horseradish peroxidase-linked secondary antibody and ECL detection (Amersham).

Measurement of GRK Activity

The membrane fractions of the myocardial extracts were used to determine GRK activity. Extracts (100 μg of protein) were incubated with rhodopsin-enriched rod outer-segment membranes in reaction buffer containing the following: MgCl_2 , 10 mmol/L; Tris-HCl, 20 mmol/L; ethylenediamine tetraacetic acid, 2 mmol/L; ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid, 5 mmol/L; and adenosine triphosphate (ATP), 0.1 mmol/L (containing [γ - ^{32}P]ATP), as previously described.¹⁵ After incubating in white light for 15 minutes at room temperature, reactions were quenched with ice-cold lysis buffer and centrifuged for 15 minutes at 13,000g. Sedimented proteins were resuspended in 25 μL of protein gel-loading dye and treated with 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis. Phosphorylated rhodopsin was visualized by means of autoradiography of dried polyacrylamide gels and quantified by using a Molecular Dynamics PhosphorImager.

Radioligand Binding Assays

Total β AR density (B_{\max}) was determined by incubating 25 μg of cardiac sarcolemmal membranes with a saturating concentration of iodine 125-labeled cyanopindolol and 20 $\mu\text{mol}/\text{L}$ alprenolol to define nonspecific binding. Sarcolemmal membrane samples were studied in triplicate with 80 pmol/L iodine 125-labeled cyanopindolol and 10^{-4} mol/L isoproterenol in 250 μL of binding buffer (50 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid [pH 7.3], 5 mmol/L MgCl_2 , and 0.1 mmol/L ascorbic acid). Assays were performed at 37°C for 1 hour and then filtered over GF/C glass fiber filters (Whatman) that were washed twice and counted in a gamma counter. Data were analyzed by means of nonlinear least-square curve fit (GraphPad Prism).

Sarcolemmal Membrane Adenylyl Cyclase Activity

Cardiac sarcolemmal membranes (20 μg of protein) were incubated for 15 minutes at 37°C with [α - ^{32}P]ATP under basal conditions, 10^{-4} mol/L isoproterenol, or 10 mmol/L NaF. Cyclic

TABLE 1. Clinical profile for patients in this study

Group	M/F	Age (y)	DCM/ICM	Drugs
NF (n = 8)	4/4	42 ± 4	—	Dopamine
HF (n = 12)	8/4	57 ± 6	5/7	Milrinone, carvedilol
LVAD (n = 12)	8/4	58 ± 5	5/7	AI

DCM, Dilated cardiomyopathy; ICM, ischemic cardiomyopathy; NF, non-failing; HF, medically managed chronic heart failure; LVAD, after left ventricular assist device implantation; AI, angiotensin-converting enzyme inhibitor.

adenosine monophosphate (cAMP) production was quantified by using standard methods described previously.¹⁷

Statistical Analysis

Repeated-measures analysis of variance (ANOVA) was used to analyze serial data over time within treatment groups. Analyses were conducted with Statview 4.01 software (Abacus Concepts Inc). Experimental groups were compared by using the Student *t* test or 1-way ANOVA, as appropriate. The Bonferroni test was applied to all significant ANOVA results by using SigmaStat software. All results are expressed as means ± standard error of the mean.

Results

Patient Population

Patient demographics are summarized in Table 1. All 12 patients underwent implantation of the HeartMate XVE LVAS during hospitalization for decompensated HF. All patients were receiving intravenous inotropic support with milrinone before LVAD insertion. Six patients were receiving a β -blocker before LVAD implantation. None were receiving this therapy after LVAD implantation. Four patients received intra-aortic balloon pumping before LVAD implantation. The indication for LVAD implantation in all patients was as a bridge to transplantation. No patients required inotropic support for right heart dysfunction by 7 days after LVAD insertion. All patients underwent LVAD explantation and orthotopic heart transplantation after a mean of 6.4 ± 1.8 months of LVAD support. All patients had New York Heart Association class IV HF symptoms at the time of LVAD implantation and improved to New York Heart Association class I by the time of heart transplantation. Normal left ventricular tissue was harvested from organ donors when the heart was not procured, despite satisfactory ventricular function.

Myocardial β AR Signaling

We assessed β AR-effector coupling in sarcolemmal membranes prepared from left ventricular apical tissue in the HF, LVAD, and nonfailing (NF) groups by measuring adenylyl cyclase (AC) activity (Figure 1). Basal AC activity was significantly lower in the HF group compared with that in the NF group (32.4 ± 1.4 vs 58.4 ± 2.0 pmol cAMP \cdot mg⁻¹ \cdot min⁻¹, *P* < .001). Isoproterenol-stimulated AC

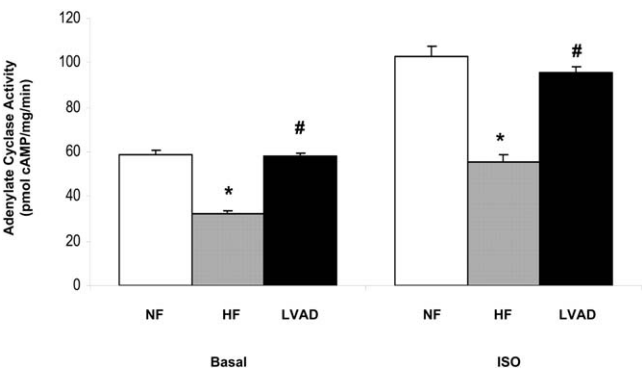


Figure 1. Myocardial sarcolemmal membrane adenylyl cyclase activity in the nonfailing (NF), heart failure (HF), and left ventricular assist device (LVAD) groups. ISO, 10⁻⁴ mol/L isoproterenol. NF group, n = 8; HF and LVAD groups, n = 12. **P* < .001 versus NF group. #*P* < .001 versus HF group and *P* > .40 versus NF group.

activity was also significantly blunted in the HF group compared with that in the NF group (55.7 ± 3.0 vs 103.1 ± 4.25 pmol cAMP \cdot mg⁻¹ \cdot min⁻¹, *P* < .001), indicating severe uncoupling of myocardial β ARs. AC activity stimulated by sodium fluoride, which induces maximal stimulation of the α subunit of the AC-stimulatory G protein, G_s, was similar in both the HF and NF groups (231.5 ± 22.7 vs 262.5 ± 19.8 pmol cAMP \cdot mg⁻¹ \cdot min⁻¹, *P* > .3), indicating that the defect in β AR signaling in the HF group is occurring at the receptor-G protein level. After LVAD support, there was a significant increase in basal AC activity compared with that in the HF group (57.8 ± 1.8 vs 32.4 ± 1.4 pmol cAMP \cdot mg⁻¹ \cdot min⁻¹, *P* < .001). There was also greater isoproterenol-stimulated AC activation after LVAD support compared with that in the HF group (95.3 ± 2.8 vs 55.6 ± 3.0 pmol cAMP \cdot mg⁻¹ \cdot min⁻¹, *P* < .001). β AR signaling is severely impaired in the chronic HF group compared with that seen in the NF control group. Basal and β -agonist-stimulated AC activities were markedly improved after LVAD support compared with those in the HF group and not statistically different from those in the NF control group. These data indicate that mechanical unloading with an LVAD can restore left ventricular β AR coupling to AC to a degree that is similar to that seen in NF control subjects.

Myocardial β AR Density and G Protein Expression

B_{max} was measured in all groups by means of radioligand binding (Figure 2). B_{max} was significantly lower in the HF group compared with that in the NF group (41.5 ± 2.0 vs 80.3 ± 1.8 fmol/mg membrane protein, *P* < .001). B_{max} was increased after LVAD support and was significantly greater than in the HF group (77.3 ± 2.0 vs 41.5 ± 2.0 fmol/mg, *P* < .004). B_{max} was similar between the LVAD

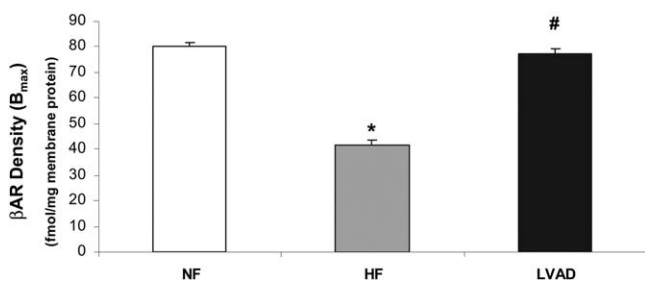


Figure 2. Myocardial sarcolemmal membrane β -adrenergic receptor (β AR) density. B_{max} , Total β AR density; NF, nonfailing group; HF, heart failure group; LVAD, left ventricular assist device group. * $P < .001$ versus NF group. # $P < .004$ versus HF group and $P > .10$ versus NF group.

and NF groups (77.3 ± 2.0 vs 80.3 ± 1.8 fmol/mg, $P = .14$). LVAD support led to upregulation of total left ventricular β AR density, which was not significantly different from that seen in the NF control group.

Left ventricular sarcolemmal membrane G protein expression was also quantified by means of Western analysis. There was no difference in expression of the AC-stimulatory G protein ($G_{\alpha s}$) among the HF, LVAD, and NF groups (data not shown). Expression of $G_{\alpha i}$, the AC-inhibitory G protein, was significantly increased in the HF group compared with the NF control group (23.8 ± 2.1 vs 10.6 ± 2.8 densitometry units [DU], $P < .001$). LVAD support led to a significant decrease in left ventricular membrane $G_{\alpha i}$ expression compared with that in the HF group (6.6 ± 3.0 vs 23.8 ± 2.1 DU, $P < .005$) and was not different from that seen in the NF control group (Figure 3).

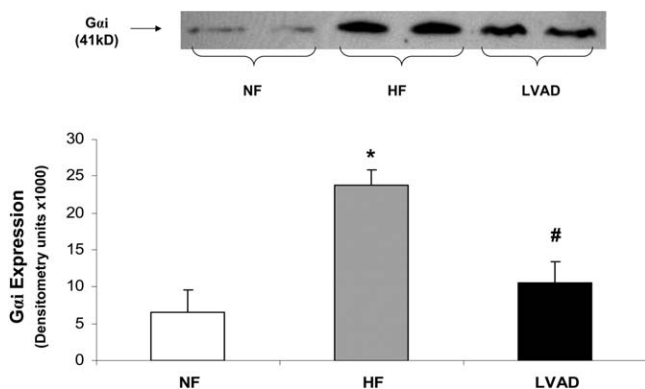


Figure 3. Left ventricular protein expression of the adenylyl cyclase-inhibitory G protein ($G_{\alpha i}$). NF, Nonfailing group; HF, heart failure group; LVAD, left ventricular assist device group. * $P < .001$ versus NF group. # $P < .005$ versus HF group and $P > .20$ versus NF group.

Myocardial GRK Expression and Activity

To determine another potential mechanism for the improvement in left ventricular β AR functional coupling after LVAD support, we studied the expression and activity of the primary GRK in the heart, GRK2 (also known as β ARK1). Left ventricular expression of GRK2 was increased 2.5-fold in the HF group compared with the NF control group (23.9 ± 2.6 vs 7.6 ± 1.6 DU, $P < .01$). GRK2 expression was significantly lower in the LVAD group compared with that in the HF group (10.1 ± 0.88 vs 23.9 ± 2.6 DU, $P < .001$) and similar to the level of expression in the NF group (10.1 ± 0.88 vs 7.6 ± 1.6 DU, $P = .07$; Figure 4, A). GRK2 activity was assessed by using a rhodopsin phosphorylation assay, and the results were similar to the expression data (Figure 4, B). GRK2 activity was increased 3-fold in the HF group compared with that in the

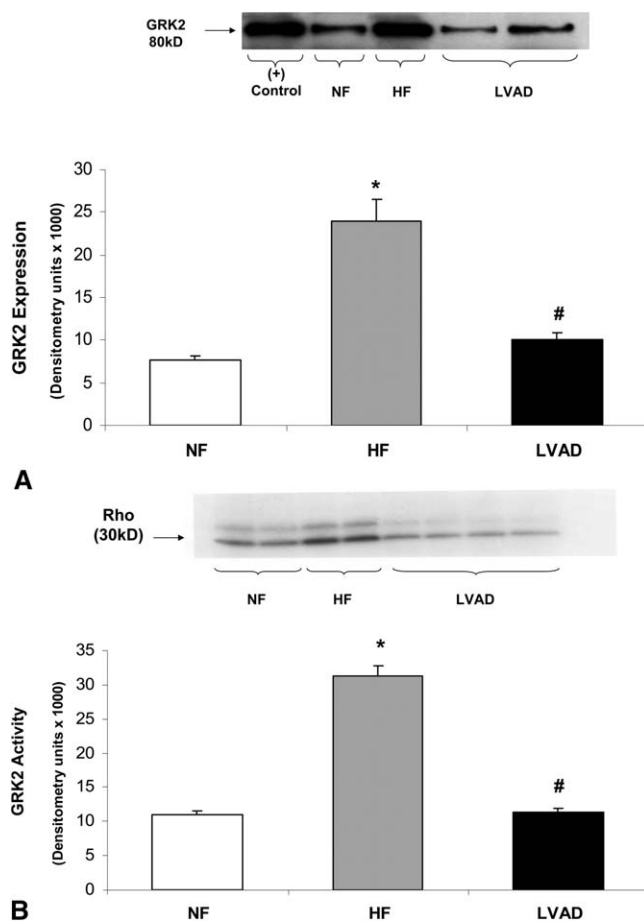


Figure 4. A, Left ventricular protein expression of G protein-coupled receptor kinase-2 (GRK2). * $P < .01$ versus NF group. # $P < .001$ versus HF group and $P > .10$ versus NF group. B, Left ventricular GRK2 activity. * $P < .004$ versus NF group. # $P < .005$ versus HF group and $P > .30$ versus NF group. NF, Nonfailing group; HF, heart failure group; LVAD, left ventricular assist device group.

NF control group (31.3 ± 1.7 vs 10.9 ± 3.6 DU, $P < .004$). After LVAD support, GRK2 activity was decreased to the level seen in the NF control group (11.4 ± 3.3 vs 10.9 ± 3.6 DU, $P = .3$).

Discussion

The myocardial β AR signaling pathway plays a critical role in the regulation of cardiac contractility. β ARs (β_1 and β_2 subtypes) are the primary myocardial targets of the sympathetic neurotransmitter norepinephrine and the adrenal hormone epinephrine. Activation of β ARs in the heart by these 2 catecholamines leads to positive chronotropic and inotropic action through stimulation of adenylyl cyclase and subsequent increases in cAMP and intracellular Ca^{2+} release.⁹ Continued exposure of β ARs to agonists results in a rapid decrease in responsiveness, which is known as desensitization.⁷ Agonist-dependent desensitization can be initiated by the phosphorylation of activated receptors by members of the family of GRKs.¹² GRK2 (or β ARK1) is a GRK that specifically phosphorylates activated β_1 - and β_2 -ARs, leading to desensitization in vitro and in vivo.^{12,15,18}

HF in human subjects has been characterized by specific alterations in the β AR signaling system. These include selective downregulation of β_1 -ARs by approximately 50% and desensitization of the remaining β ARs, which leads to the blunting of further agonist-mediated stimulation.^{7,19} The enhanced desensitization of myocardial β ARs is likely due, in large part, to the increased expression and activity of GRK2 (approximately 3-fold) present in human HF.^{10,11} It is generally thought that these changes in the β AR system in HF are triggered by increased sympathetic stimulation of the heart in this disease state.²⁰ The dysfunctional β AR signaling, including increased GRK2 expression and activity, is a contributing factor to the impaired myocardial contractility seen in HF.

LVAD support has been shown to have many beneficial effects as a result of mechanical unloading. These include decreasing heart size,^{21,22} improvement in ventricular function,² decreased plasma catecholamines^{1,23} and cytokines,²⁴ and decreased ventricular expression of atrial natriuretic peptide²⁵ and tumor necrosis factor α .²⁶ Recent studies have also demonstrated an improvement in left ventricular β AR signaling after long-term LVAD support.^{13,14} These studies have shown that LVAD support can lead to upregulation of myocardial β AR density to near normal and restore ex vivo cardiac muscle contraction in response to β -agonist stimulation. It was thought that this restoration of β AR signaling in the heart was primarily due to hemodynamic factors resulting from unloading of the left ventricle. More recently, it has been demonstrated that there is significant improvement in β AR signaling in both the left ventricle and the right ventricle after LVAD support.¹⁴ This study suggested that restoration of the myocardial β AR signaling pathway

during LVAD support is primarily mediated by systemic factors, such as biochemical milieu, and is not directly mediated by hemodynamic factors.

The primary objective of the present study was to determine the role of GRK activity in the restoration of left ventricular β AR signaling after LVAD support. We hypothesized that the relative normalization of the neurohormonal milieu resulting from mechanical unloading^{1,23} and significantly improved cardiac hemodynamics would lead to a decrease in GRK2 expression and activity. This would allow for the restoration of β AR density to near normal and would enhance coupling through these receptors as a result of a lower degree of GRK-mediated desensitization.

As others have reported,^{13,14} we also found that long-term LVAD support can restore myocardial β AR signaling, as demonstrated by increasing sarcolemmal membrane β AR density to near normal. Basal and β -agonist stimulated adenylyl cyclase activity was also restored to normal during LVAD support. The expression and activity of GRK2, the primary GRK in the heart, were reduced by nearly 3-fold to normal levels in the LVAD group. In addition, there was a significant reduction in left ventricular expression of G_i , the cyclase inhibitory G protein, as a result of LVAD support, which likely plays a role in the normalization of adenylyl cyclase activity and improved myocyte contractility.

The critical role of GRK2 as a mediator of cardiac function has been well described. Transgenic mice with cardiac-specific overexpression (3-fold) of GRK2 have severely blunted basal and β -agonist-stimulated left ventricular function.¹⁵ In contrast, mice with cardiac-specific expression of a peptide inhibitor of GRK2 have significantly enhanced basal and agonist-stimulated cardiac contractility because desensitization of myocardial β ARs is diminished.¹⁵ β -Adrenergic-based gene therapy approaches using an inhibitor of GRK2 activity have also been successful in restoring β AR signaling in failing cardiac myocytes,²⁷ as well as improving cardiac function in animal models of myocardial infarction and HF.^{28,29} A recent report demonstrated that increased cardiac GRK2 levels correlate with decreased β AR signaling in failing hearts.³⁰ The study also suggested that increased cardiac GRK2 levels might be associated with poorer cardiac function or clinical signs of HF. Another important finding in this study was the direct demonstration that cardiac GRK2 levels can be monitored by measuring peripheral lymphocyte GRK2 expression and activity.³⁰

Our study is the first to demonstrate that myocardial GRK2 expression and activity can be modulated or inhibited in human HF. Long-term LVAD support led to a significant decrease in cardiac GRK2 levels of expression and activity compared with medical therapy and resulted in restoration of left ventricular β AR signaling. There was a significant upregulation of β AR density, a decrease in $G_{\alpha i}$

expression, and an increase in basal and β -agonist-stimulated cAMP production. The decrease in cardiac GRK2 levels might be due to relative normalization of the neurohormonal milieu after the improved hemodynamic status of these patients with HF who were receiving LVAD support. Monitoring peripheral lymphocyte GRK2 levels, which appear to correlate with cardiac GRK2 activity, after LVAD support might provide important additional clinical data in using LVADs as a bridge to myocardial recovery in the setting of chronic HF. Restoration of myocardial β AR signaling through inhibition of GRK2 activity in human HF might represent a novel therapeutic strategy for this disease.

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